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Evolution of Swine H3N2 Influenza Viruses in the United States

RICHARD J. WEBBY,[1] SABRINA L. SWENSON,[2] SCOTT L. KRAUSS,[1] PHILIP J. GERRISH,[3] SAGAR M. GOYAL,[4] AND ROBERT G. WEBSTER[1,5]*

Department of Virology and Molecular Biology,[1] St. Jude Children's Research Hospital, Memphis, Tennessee 38105; National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, Iowa 50010[2]; Department of Theoretical Biology and Biophysics, Los Alamos National Laboratory, Los Alamos, New Mexico 87545[3]; Veterinary Diagnostic Medicine, University of Minnesota, St. Paul, Minnesota 55108[4]; and Department of Pathology, University of Tennessee, Memphis, Tennessee 38163[5]

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During 1998, severe outbreaks of influenza were observed in four swine herds in the United States. This event was unique because the causative agents, H3N2 influenza viruses, are infrequently isolated from swine in North America. Two antigenically distinct reassortant viruses (H3N2) were isolated from infected animals: a double-reassortant virus containing genes similar to those of human and swine viruses, and a triple-reassortant virus containing genes similar to those of human, swine, and avian influenza viruses (N. N. Zhou, D. A. Senne, J. S. Landgraf, S. L. Swenson, G. Erickson, K. Rossow, L. Liu, K.-J. Yoon, S. Krauss, and R. G. Webster, J. Virol. 73:8851–8856, 1999). Because the U.S. pig population was essentially naive in regard to H3N2 viruses, it was important to determine the extent of viral spread. Hemagglutination inhibition (HI) assays of 4,382 serum samples from swine in 23 states indicated that 28.3% of these animals had been exposed to classical swine-like H1N1 viruses and 20.5% had been exposed to the triple-reassortant-like H3N2 viruses. The HI data suggested that viruses antigenically related to the double-reassortant H3N2 virus have not become widespread in the U.S. swine population. The seroreactivity levels in swine serum samples and the nucleotide sequences of six additional 1999 isolates, all of which were of the triple-reassortant genotype, suggested that H3N2 viruses containing avian PA and PB2 genes had spread throughout much of the country. These avian-like genes cluster with genes from North American avian viruses. The worldwide predominance of swine viruses containing an avian-like internal gene component suggests that these genes may confer a selective advantage in pigs. Analysis of the 1999 swine H3N2 isolates showed that the internal gene complex of the triple-reassortant viruses was associated with three recent phylogenetically distinct human-like hemagglutinin (HA) molecules. Acquisition of HA genes from the human virus reservoir will significantly affect the efficacy of the current swine H3N2 vaccines. This finding supports continued surveillance of U.S. swine populations for influenza virus activity.

Influenza viruses have been isolated from a number of different animal hosts including birds, humans, horses, whales, minks, and pigs. Generally, influenza viruses are host specific and viruses from one host rarely establish stable lineages in another host species. Although whole viruses may rarely transmit, gene segments can cross the species barrier through the process of genetic reassortment. Pigs have been postulated to play an important role in the process of genetic reassortment by acting as the "mixing vessel" for such events (27). Pigs, unlike humans (1), seem to be readily infected by avian viruses, and most, if not all, avian HA subtypes are capable of replicating in swine (18). A molecular mechanism has been proposed for the susceptibility of swine to avian virus infection. The viral receptor sialyloligosaccharides that are present on the pig tracheal cells possess both *N*-acetylneuraminic acid-α2,3-galactose (NeuAcα2,3Gal) and NeuAcα2,6Gal linkages (17). Human influenza viruses preferentially bind NeuAcα2,6Gal linkages, whereas avian influenza viruses bind NeuAcα2,3Gal linkages (25). Thus, pig tracheal cells can be infected not only by human influenza viruses but also by avian influenza viruses. The direct chicken-to-human transmission of H5N1 viruses in Hong Kong during 1997, however, argues that factors in addition to receptor specificity must be involved in influenza virus interspecies transmission (10, 30, 31).

Influenza in swine is an acute respiratory disease whose severity depends on many factors including pig age, virus strain, and secondary infections (14). Currently three main subtypes of influenza virus are circulating in different swine populations throughout the world: H1N1, H3N2, and H1N2. In Asia, North America, and much of Europe, viruses of the H1N1 subtype are the most commonly isolated (4, 28). The circulating H1N1 viruses differ, however, in the origins of their genomic components. The H1N1 viruses in North America and Asia belong to the classic swine lineage, which is genetically related to the human H1N1 viruses responsible for the 1918 Spanish influenza pandemic (24, 29, 32). In contrast, all eight genes of the H1N1 virus circulating in Europe are phylogenetically related to the avian lineage (29). The avian-like H1N1 virus is also present in the United Kingdom, although the virus of current concern is a reassortant H1N2 virus with gene segments derived from both human and avian lineages (3). Viruses of the H3N2 subtype circulate in Asia and Europe but have been infrequently isolated in North America (8, 16, 28). Before 1998, the most recent isolation of an H3N2 virus in North America occurred in Canada in 1991. The hemagglutinin (HA) molecule of this Canadian isolate was similar to that of the human virus A/Victoria/3/75 (2).

In late August 1998, a severe influenza-like illness was observed in pigs on a farm in North Carolina. During November and December of the same year, additional outbreaks among swine herds were reported in Minnesota, Iowa, and Texas. The

* Corresponding author. Mailing address: Department of Virology and Molecular Biology, St. Jude Children's Research Hospital, 332 N. Lauderdale St., Memphis, TN 38105. Phone: (901) 495-3400. Fax: (901) 523-2622. E-mail: robert.webster@stjude.org.

TABLE 1. Specificity of antigens used in the swine serologic survey

Isolate	Reciprocal HI titer for antiserum against[a]:						
	A/Swine/Iowa/15/30 (H1N1)	A/Swine/Texas/4199-2/98 (H3N2)				A/Swine/North Carolina/35922/98 (H3N2)	
		A	B	C	D	A	B
A/Swine/Iowa/3421/90 (H1N1)	160	<10	<10	<10	<10	<10	<10
A/Swine/Texas/4199-2/98 (H3N2)	<10	640	640	1,280	1,280	<10	10
A/Swine/North Carolina/35922/98 (H3N2)	<10	10	10	20	20	320	640
A/Shorebird/Delaware/28/95 (H7N9)	<10	<10	<10	<10	<10	<10	<10

[a] A to D indicates that serum was obtained from four animals infected with A/Sw/TX/98 and two infected with A/Sw/NC/98.

causative agents were subsequently identified as influenza viruses of the subtype H3N2. Genetic analysis of these H3N2 viruses showed that at least two different genotypes were present. The initial North Carolina isolate contained gene segments similar to those of the human (HA, NA, and PB1) and classic swine (NS, NP, M, PB2, and PA) lineages, whereas the isolates from Minnesota, Iowa, and Texas contained genes from the human (HA, NA, and PB1), swine (NS, NP, and M), and avian (PB2 and PA) lineages (39).

The severity of disease and the isolation of H3N2 influenza viruses in swine are issues of concern for the ecology and epidemiology of influenza in the United States. The isolation of viruses from four states indicates that H3N2 viruses have spread and could become permanently established in U.S. swine. To determine the extent of virus distribution and to address other issues concerning the impact of these viruses, we performed a widespread serologic survey of U.S. swine populations. The genetic composition of six recently isolated swine H3N2 influenza viruses was also determined.

MATERIALS AND METHODS

Virus strains. All viruses used in this study were obtained from the National Veterinary Services Laboratories, Ames, Iowa, and from the repository at St. Jude Children's Research Hospital, Memphis, Tenn. When necessary, viruses were grown in the allantoic cavities of 11-day embryonated chicken eggs before RNA extraction or antigenic analysis was performed.

Serum collection. Pig serum samples were collected from North American swine herds in collaboration with the National Veterinary Services Laboratories, state veterinary diagnostic laboratories, and state veterinarians. Samples were taken from a cross-section of the pig population including animals of different breeds, ages, and sexes. Most samples were collected during late 1998 and early 1999 and originated from swine in the central and eastern states; this predominance reflects the significance of this region in U.S. pork production. In situations where the pig population was sufficiently large, 20 serum samples were collected from each of 10 herds within a state. A total of 4,382 serum samples from swine in 23 states were collected and tested.

Serologic testing. The collected sera were tested for the presence of antibodies to influenza virus surface glycoproteins by the hemagglutination inhibition (HI) test as previously described (21). All sera were pretreated with the receptor-destroying enzyme from *Vibrio cholerae* (Denka Seiken, Tokyo, Japan) to abolish interference by nonspecific serum inhibitors. The antigens used in the HI assay were A/Swine/Iowa/3421/90 (Sw/IA/90 [H1N1]), A/Swine/Texas/4199-2/98 (Sw/TX/98 [H3N2]), A/Swine/North Carolina/35922/98 (Sw/NC/98 [H3N2]), and A/Shorebird/Delaware/28/95 (SB/DE/95 [H7N9]), which was used as a negative control. These viruses, which came directly from infected allantoic fluid, were diluted to four hemagglutination doses. For consistency with previously published studies, HI titers equal to or greater than 1:40 were recorded as being positive (i.e., as showing evidence of a previous exposure to HI antigen). Maximum-likelihood titers were computed by assuming the probability of nonreactivity to be that of the Poisson zero-class with parameter XD, where X is the titer and D is the dilution factor. A P value was defined as the probability that Sw/NC/98 titers were greater or equal to Sw/TX/98 titers. It was computed as an expected P value by multiplying by the normalized likelihood function and numerically integrating over all values of X.

RNA extraction, RT-PCR, and DNA sequencing. Viral RNA was extracted from allantoic fluid by using the RNeasy kit (Qiagen, Valencia, Calif.) as specified by the manufacturer. Reverse transcription and PCR were carried out under standard conditions by using influenza virus-specific primers. The sequences of these primers and a description of the amplification conditions are available upon request. PCR products were purified by using a QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed by the Hartwell Center for Bioinformatics and Biotechnology at St. Jude Children's Research Hospital. Template DNA was sequenced by using rhodamine or dRhodamine dye terminator cycle-sequencing ready reaction kits with AmpliTaqDNA polymerase FS (Perkin-Elmer, Applied Biosystems, Inc., Foster City, Calif.) and synthetic oligonucleotides. Samples were subjected to electrophoresis, detection, and analysis on Perkin-Elmer, Applied Biosystems model 373 or model 377 DNA sequencers.

Sequence analysis. DNA sequences were compiled and edited by using the Lasergene sequence analysis software package (DNASTAR, Madison, Wis.). Multiple-sequence alignments were made by using CLUSTAL W (33), and phylogenetic trees were generated by using the neighbor-joining algorithm within the PHYLIP version 3.57C software package (15).

Nucleotide sequence accession numbers. Sequences obtained in this study have been deposited in the GenBank database under the accession codes AF268102-AF268170.

RESULTS

Cross-reactivity of HI antigens. An objective of the present study was to determine the spread of both Sw/NC/98 and Sw/TX/98 in pig populations. If a serologic approach (i.e., an HI assay) is used to fulfill this objective, the two viruses, which serve as antigens in the proposed assay, must be antigenically distinct. Sera were obtained from pigs experimentally infected with Sw/NC/98 or Sw/TX/98. Because no specific antiserum to A/Sw/Iowa/90 was available, goat antiserum raised to A/Sw/Iowa/15/30 was used to assess the cross-reactivity of classic H1N1 viruses. The results of the HI assay show that there is no cross-reactivity between the surface antigens of H3N2 and H1N1 viruses but that the two H3N2 viruses have common epitopes (Table 1). The HI titers were, however, much higher against the homologous antigen, which confirmed that antiserum raised against Sw/TX/98 could be differentiated from antiserum raised against Sw/NC/98.

Seroprevalence of swine influenza in U.S. pig populations. The isolation of H3N2 viruses from multiple sites in 1998 suggested that these viruses might form stable lineages in U.S. swine. We undertook a serologic survey to determine whether H3N2 viruses had continued to spread and, if so, whether both viral lineages had been maintained. A collection of 4,382 pig serum samples was analyzed in HI assays for the presence of antibodies to two H3N2 antigenic variants and to classic H1N1 swine influenza virus (Table 2). The total percentages of seroreactive animals in all states were 28.3, 20.5, and 8.3% against Sw/IA/90 (H1N1), Sw/TX/98 (H3N2, triple reassortant), and Sw/NC/98 (H3N2, double reassortant), respectively. No HI was detected against the negative control virus, A/Shorebird/Delaware/28/95. H3N2-seroreactive animals were found throughout the country, with the highest levels being apparent in the central states (Fig. 1). A portion (8.3%) of the animals had detectable levels of antibodies reactive to Sw/NC/98; however, most of these antibodies were probably cross-reactive antibod-

TABLE 2. Seroprevalence of influenza viruses in U.S. swine populations

State (no. of animals)	% of total U.S. pig production[a]	% of seropositive animals as determined by HI assay			Proportion of herds with H3N2 seroreactivity[d]
		A/Swine/Iowa/ 3421/90 (H1N1)	A/Swine/Texas/ 4199-2/98[b] (H3N2)	A/Swine/NCarolina/ 35922/98[c] (H3N2)	
Iowa (202)	25.9	41.6	65.8	46.0	10/10
North Carolina (167)	16.0	38.9	13.2	7.7	ND[e]
Minnesota (200)	9.3	30.5	14.0	6.0	6/10
Illinois (200)	6.9	59.5	42.5	19.0	10/10
Indiana (400)	5.5	36.0	9.5	3.0	10/20
Missouri (200)	5.3	50.5	1.0	0	2/10
Nebraska (200)	5.0	42.0	16.5	1.0	3/10
Oklahoma (200)	3.8	33.0	56.0	24.5	9/10
Kansas (200)	2.5	22.5	26.0	8.0	9/10
South Dakota (200)	2.1	9.0	32.5	6.6	5/10
Pennsylvania (32)	1.8	75.0	37.5	12.5	ND
Michigan (200)	1.6	9.0	2.5	0	2/10
Colorado (52)	1.5	19.2	36.5	23.1	1/2
Texas (200)	1.5	57.0	52.0	26.5	7/10
Arkansas (200)	1.2	9.0	12.0	2.5	7/10
Wisconsin (400)	1.0	29.2	5.0	3.0	9/20
Georgia (160)	0.8	15.6	11.9	4.4	4/8
Virginia (100)	0.6	60.0	14.0	2.0	3/5
Tennessee (200)	0.4	2.5	18	3.5	4/10
California (257)	0.3	13.2	10.1	0	5/13
Florida (153)	0.07	11.8	15.0	5.9	ND
Massachusetts (145)	0.04	8.3	12.4	0.7	ND
New Jersey (114)	0.03	0	7.8	0.9	ND
Total (4,382)	93.1	28.3	20.5	8.3	

[a] Values obtained from December Hogs and Pigs report, National Statistics Service, U.S. Department of Agriculture.
[b] Triple-reassortant virus.
[c] Double-reassortant virus.
[d] Number positive/number tested.
[e] ND, not determined.

ies directed against other H3 viruses because only 13 (0.3%) of the tested samples had higher HI titers against Sw/NC/98 than against Sw/TX/98. The probability of Sw/NC/98-seropositive animals having higher HI titers against Sw/TX/98 was determined by calculating the maximum-likelihood values derived from the HI data from all Sw/NC/98 positive samples. The calculated maximum-likelihood titers were 62.7 and 127.5 for Sw/NC/98 and Sw/TX/98, respectively. This difference was

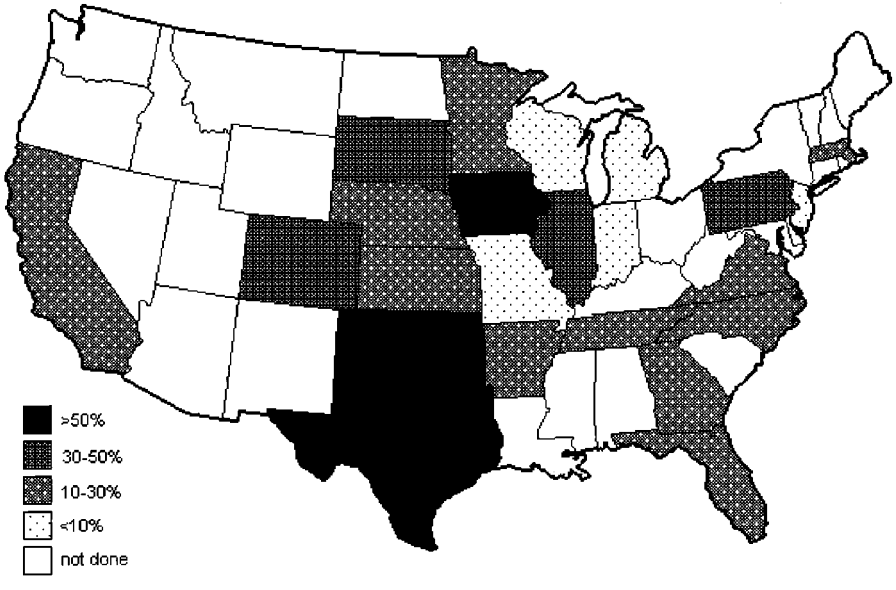

FIG. 1. Map of the United States showing the distribution of H3N2-seropositive animals. The shading represents the percentage of Sw/TX/98-seroreactive animals in each state.

TABLE 3. Comparison of the nucleotide sequences of the internal genes of swine H3N2 viruses in the United States

Virus	Nucleotide similarities (%) to Sw/TX/98 and Sw/NC/98					
	PB1 (26–507)[a]	PB2 (1000–1469)	PA (768–1224)	NP (68–561)	NS (22–523)	M (52–337)
Sw/CO/23619/99	99 and 97	99 and 80	99 and 77	99 and 95	99 and 98	98 and 93
Sw/OK/18717/99	99 and 97	99 and 80	99 and 76	99 and 95	99 and 98	99 and 94
Sw/OK/18089/99	99 and 97	99 and 80	99 and 76	99 and 95	99 and 98	99 and 94
Sw/WI/14094/99	99 and 97	99 and 80	99 and 76	99 and 95	99 and 98	99 and 94
Sw/IL/21587/99	99 and 97	99 and 80	99 and 76	99 and 95	99 and 98	99 and 94
Sw/NC/16497/99	99 and 97	99 and 80	99 and 77	99 and 95	99 and 98	98 and 94

[a] Nucleotide positions of the region compared.

highly significant ($P < 0.001$). A total of 9.8% of the animals had reactive antibodies against both H3N2 and H1N1 antigens. Although there is no evidence that the H1N1 and H3N2 infections in these animals were concomitant or that some of the H1N1 seropositivity was due to vaccination, the double reactivity raises the possibility that further genetic reassortment may occur in pig populations. The highest incidence of animals seropositive for both H1N1 and H3N2 viruses was found in Illinois (30.5%), Texas (38.5%), and Oklahoma (24.5%), suggesting that swine in these states warrant further monitoring for the emergence of novel reassortant viruses.

Characterization of genes encoding internal proteins in the 1999 H3N2 swine isolates. The results of the serologic survey showed an increase in the number of H3N2-seropositive pigs in the United States. To determine whether this increase is due to the spread of single or multiple lineages of virus, we determined the genotypes of a collection of H3N2 viruses isolated from swine in Oklahoma, Illinois, Colorado, Wisconsin, and North Carolina during 1999. Partial sequencing of the gene segments encoding internal proteins showed that all six sequenced viruses had the same genotype as the triple-reassortant viruses Sw/TX/98, Sw/MN/98, and Sw/IA/98 (Table 3). Therefore, viruses of the triple-reassortant genotype have now been isolated from at least eight states throughout the central and eastern United States. No additional isolates with the double-reassortant gene constellation were found, a finding supporting our contention that much of the seroreactivity to Sw/NC/98 was due to cross-reactivity.

Characterization of genes encoding surface proteins in the 1999 H3N2 swine isolates. The internal genes of the 1998 and 1999 triple-reassortant viruses displayed little variation (Table 3). These gene segments, however, are not subject to selective pressure exerted by the host immune response, such as that exerted on the surface glycoproteins. Because of the continual availability of naive animals, the immunologic pressure in swine populations is considered to be less severe than that in humans. We compared the antigenic relationship between Sw/TX/98 and the 1999 H3N2 swine isolates in an HI assay. The 1999 isolates were antigenically heterogeneous (Fig. 2). The HA1 region of each 1999 isolate was partially sequenced, and the sequences were compared with those of the 1998 swine isolates and recent human H3N2 strains. A phylogenetic tree

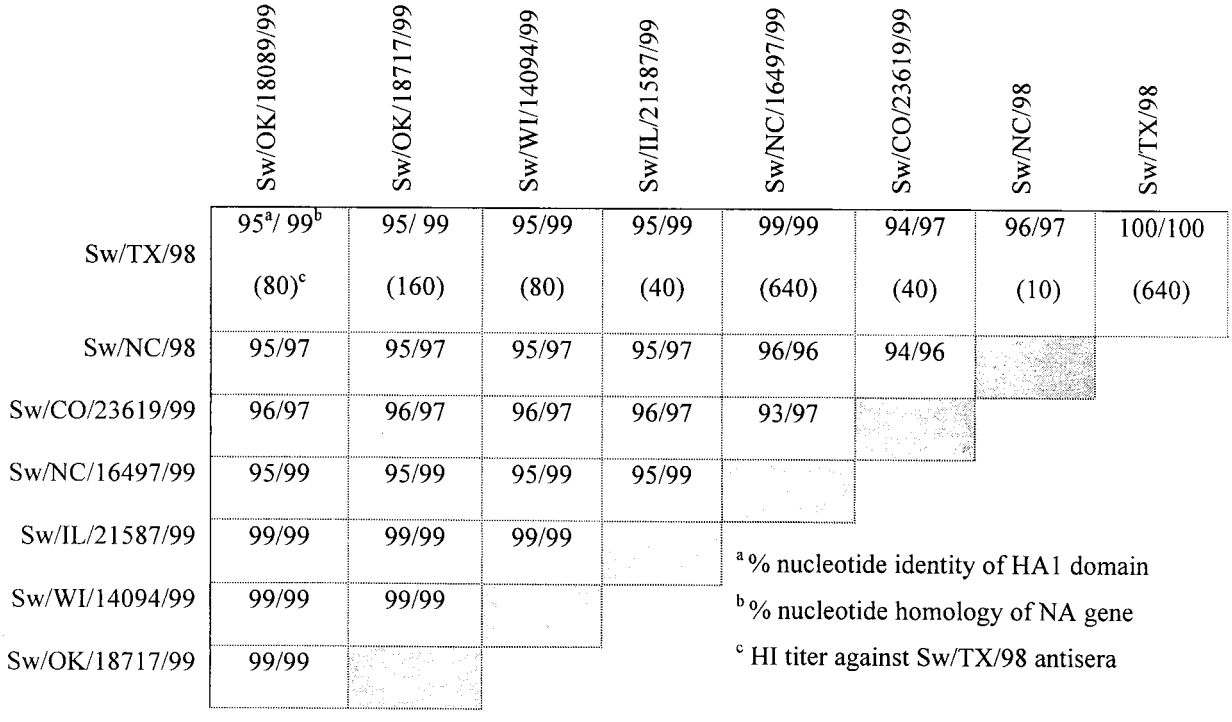

FIG. 2. Comparison of the antigenicity and of the HA1 and NA nucleotide sequences of U.S. swine H3N2 viruses.

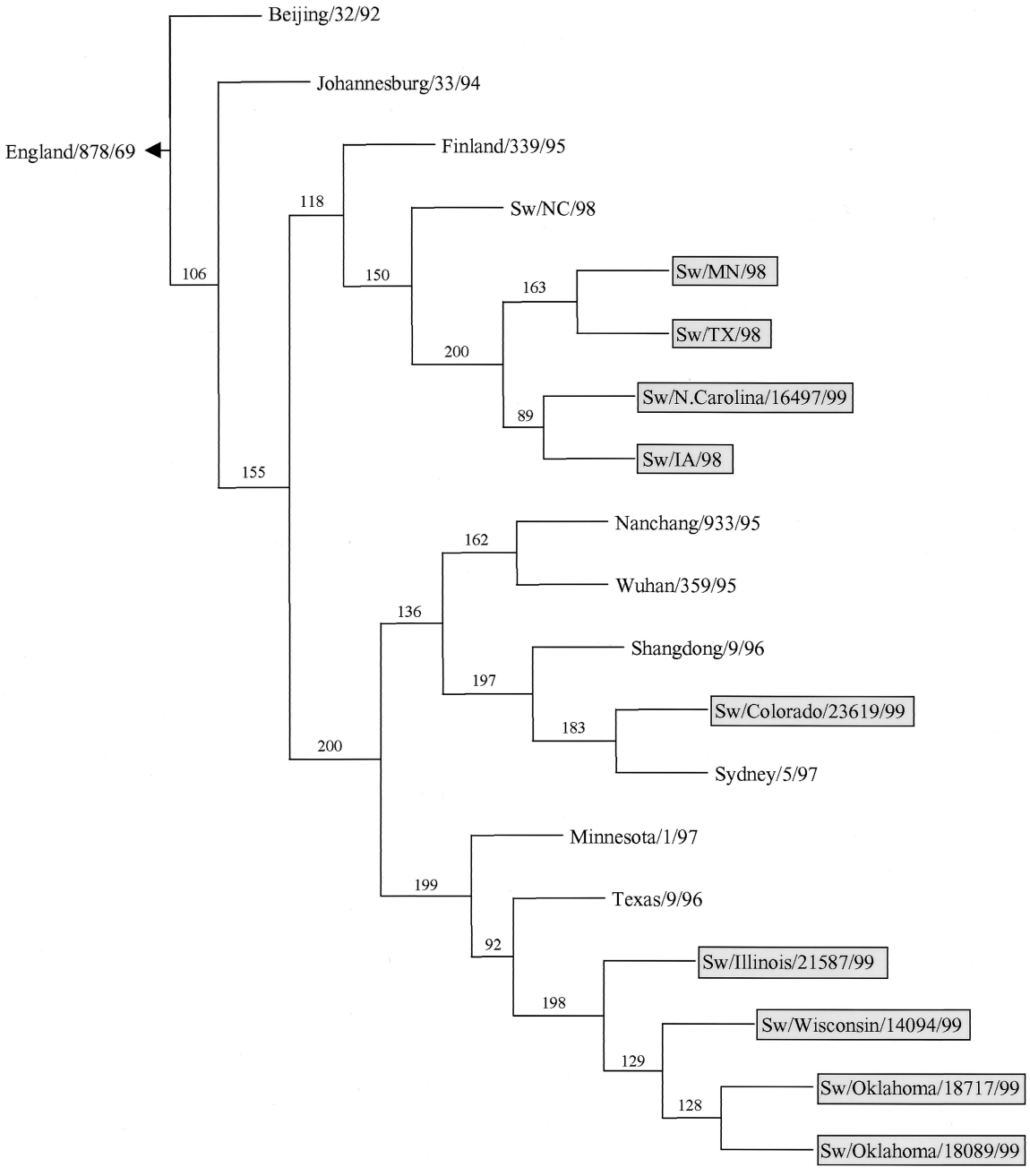

FIG. 3. Phylogenetic tree based on the nucleotide sequences of the HA1 genes of selected H3N2 influenza viruses. The boxed isolates are swine viruses that have the triple-reassortant genotype. With the exception of Sw/NC/98 (a double-reassortant virus), all remaining viruses were isolated from humans. Horizontal distances are proportional to the genetic distance, and the numbers above the nodes are bootstrap scores out of 200 replicates.

produced from these data (Fig. 3) shows that three distinct clusters of HA molecules are associated with viruses of the triple-reassortant genotype. The swine viruses do not form a separate lineage, and the HA genes from each swine cluster are more closely related to those of circulating human strains than to those of the swine viruses of the other clusters. This finding suggests that the triple-reassortant swine viruses have undergone reassortment with human H3N2 viruses on at least three occasions. Nucleotide identities between the HA genes of the 1999 swine isolates and Sw/TX/98 ranged from 94% (Sw/CO/23619/99) to 99% (Sw/NC/16497/99) (Fig. 2). The highest GenBank similarities to the HA of Sw/CO/23619/99 were found for A/Sydney/5/97-like viruses, with Sw/WI/14094/99, Sw/OK/18089/99, Sw/IL/21587/99, and Sw/OK/18717/99 being most similar to those of human viruses circulating in the United States in 1996. The HA1 sequences of the 1996 U.S. viruses were more similar to those of A/Wuhan/359/95-like viruses than to those of A/Sydney/5/97-like. A/Wuhan/359/95-like viruses were the predominant H3N2 virus isolated in the United States before the 1997 to 1998 influenza season; during that season, the A/Sydney/5/97-like viruses were the influenza virus most commonly isolated from humans (6, 7).

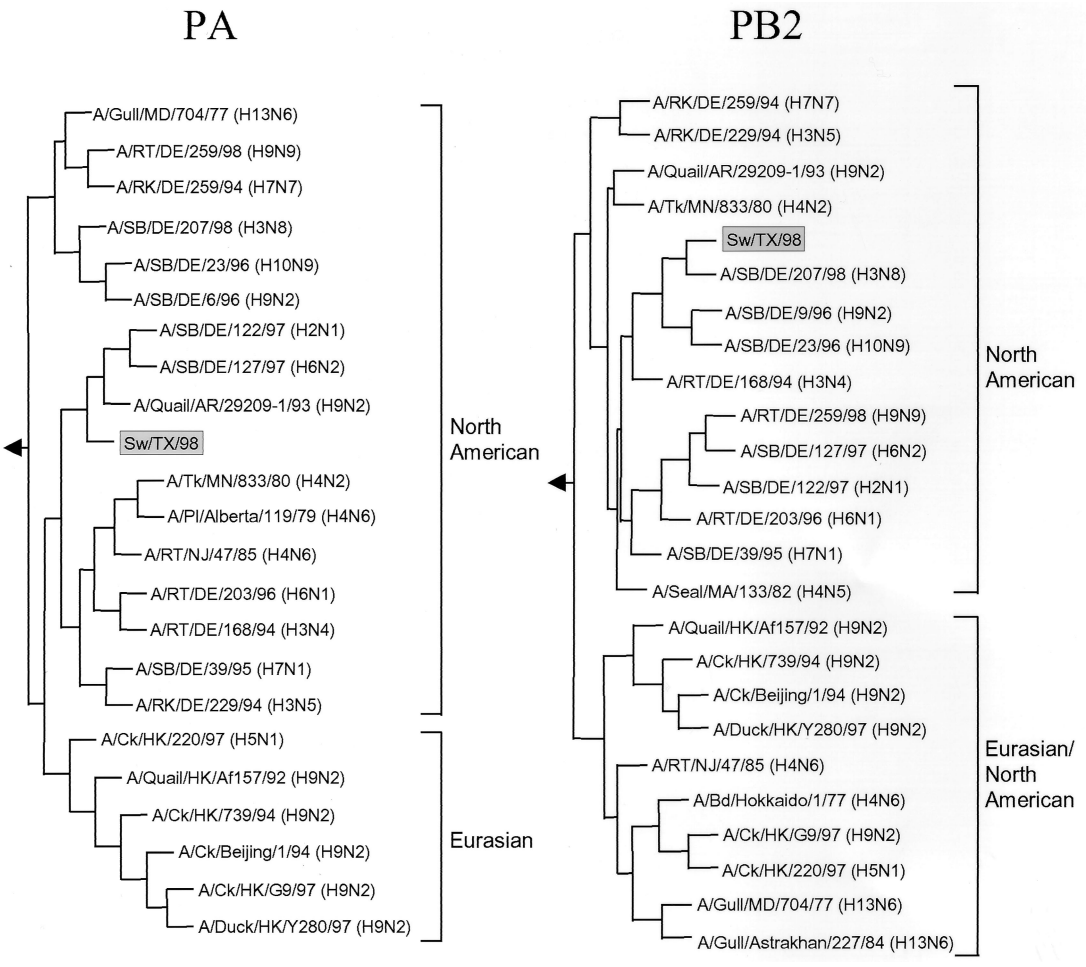

FIG. 4. Phylogenetic trees produced from the partial nucleotide sequence of the PA and PB2 genes from Sw/TX/98 and a collection of avian influenza viruses. Horizontal distances are proportional to the genetic distance. RK, Red Knot; RT, Ruddy Turnstone; SB, unidentified shorebird; Ck, chicken; Tk, turkey; Pl, pintail; Bd, budgerigar; DE, Delaware Bay; MD, Maryland; MA, Massachusetts; NJ, New Jersey; AR, Arkansas; MN, Minnesota; HK, Hong Kong.

The NA genes of the 1999 swine viruses were also sequenced (Fig. 2). The NA genes were more conserved than the HA genes, although the unavailability of sequence data for recently isolated North American human strains made phylogenetic analysis of the NA genes difficult. Therefore, we were unable to determine whether the reassortment events resulted in the acquisition of human HA genes or both human HA and NA genes by the swine viruses.

Origin of the triple-reassortant avian genes. Viruses of the triple-reassortant genotype have spread throughout the country, whereas viruses of the double-reassortant genotype have not. Although there is some sequence divergence in the swine and human-like genes of the triple and double-reassortant viruses, it is interesting to speculate that possession of the avian gene complement (i.e., the PA and PB2 genes) confers a selective advantage. To further characterize the avian-like PA and PB2 genes, we attempted to identify the possible donor reservoir of these genes. The GenBank entry with the greatest nucleotide sequence homology to the PA gene of Sw/TX/98 was the A/Quail/Arkansas/29209-1/93 sequence (H9N2; 98% identity). The PB2 nucleotide sequence of Sw/TX/98 was most similar to that of A/Shorebird/Delaware/9/96 (H9N2; 97% identity). The homology between the PB2 genes suggests that shorebird viruses could be the source of the avian-like genes. In response to this possibility, we determined the nucleotide sequence of regions of the PA (nucleotides 800 to 1229) and PB2 (nucleotides 1005 to 1470) genes from randomly selected viruses isolated from migratory shorebirds in the Delaware Bay region between 1994 and 1998. The nucleotide sequences of some PA and PB2 genes of the shorebird viruses were similar to those of Sw/TX/98 (Fig. 4). Although there was no clear separation of all Eurasian and North American isolates, the triple-reassortant PA and PB2 genes were clustered with avian viruses isolated in North America. This finding suggests that the avian-like swine genes belong to a North American avian lineage and that the reassortment event between the parental viruses occurred in North America.

DISCUSSION

The isolation of two distinguishable H3N2 viruses in U.S. swine populations during 1998 represented a significant event in the epidemiology of swine influenza because, unlike the situation in Asia and Europe, the isolation of H3N2 viruses from pigs in North America has been infrequent. Previous serologic studies of U.S. swine herds had identified H3N2 seroprevalence rates of 1.4% in 1976 to 1977 and of 1.1% in 1987 to 1989 (8, 16). These rates were significantly lower than

the corresponding levels of seroreactivity to H1N1 viruses (average levels of 21% in 1977 to 1978 and 30% in 1988 to 1989). In a recent study, 8% of animal sera collected in 1997 to 1998 were seropositive for H3N2 virus (20). The seroprevalence rate of 20.5% in the present study indicates that viruses antigenically related to Sw/TX/98 have spread throughout a large proportion of U.S. swine herds. The levels of H1N1 seroreactivity, at the same time, have remained relatively constant since the 1976 to 1977 survey. However, it should be noted that an H1N1 vaccine is commercially available; therefore, an unknown proportion of the animals in the present study may have tested positive for exposure to H1N1 virus because of vaccination. Additional serum samples were collected from Puerto Rico and Alaska. Only a small percentage of these samples indicated any exposure to H3N2 or H1N1 influenza viruses (data not shown).

Although the increase in H3N2 activity is due to viruses antigenically similar to Sw/TX/98, it is not certain that this increase is due to genetically similar viruses. However, the isolation of viruses of the triple-reassortant genotype from eight states during a 9-month period does indicate that viruses with the triple-reassortant genotype are responsible for the increase in H3N2 seroreactivity. Although Sw/NC/98-reactive sera were identified, collectively these animals had significantly higher HI titers against Sw/TX/98. Using the observed percentages of H3N2-reactive animals (20.5% for Sw/TX/98 and 8.3% for Sw/NC/98), the maximum expected frequency of an animal being exposed to both H3N2 viruses in a well-mixed population would be only 1.7%. Thus, the unlikely nature of mixed infection, the lack of further virus isolation, and the significantly higher titers against Sw/TX/98 all suggest that much of the Sw/NC/98 reactivity was due to cross-reactivity.

The establishment of the triple-reassortant virus is consistent with the apparent selective advantage displayed by swine viruses containing avian-like genes. In 1979, an H1N1 virus of avian origin was transmitted to pigs in northern Europe (22, 29). This virus eventually replaced the existing classic swine H1N1 as the predominant virus. A similar theme is apparent in the United Kingdom, where reassortant H1N2 viruses with internal genes of the avian lineage are becoming the main influenza viruses in swine (3, 13). In addition, of 11 H3N2 viruses analyzed in Europe between 1992 and 1995, all contained internal genes from the avian-like H1N1 swine virus (4, 5). Therefore, viruses containing avian-like genes appear to have some selective advantage and are preferentially maintained in swine populations.

As shown by the sequencing of a small sample of viruses isolated from migratory shorebirds, the avian-like genes of the swine H3N2 viruses cluster with genes from North American viruses. There is some evidence from the analysis of duck and shorebird populations that the predominant circulating influenza virus subtypes may differ in various avian reservoirs (35). Little, however, is known about the extent of internal gene variation in these birds. Although PA and PB2 genes similar to those of the triple-reassortant virus were found to circulate in migratory shorebirds, it remains to be determined whether this was the actual donor reservoir. Further analysis of viruses of different North American avian species must be performed before it can be determined whether pools of species-specific internal genes exist in birds.

One of the unexpected findings of this study is the antigenic variability of the HAs of the triple-reassortant viruses isolated in 1999. The association of the triple-reassortant internal genes with three phylogenetically distinct human HA genes suggests that selective pressures are present to drive this reassortment. The HA genes of the viruses responsible for the 1998 swine disease outbreaks were most similar to those of the 1995 human viruses (39). The 1999 swine isolates can be separated into three groups on the basis of the sequence of their HA genes. One virus, A/Sw/NC/16497/99, is similar to the initial isolates (and hence to the 1995 human strains), a second group is similar to U.S. human strains circulating in 1996, and A/Sw/CO/23619/99 is similar to A/Sydney/5/97. The similarities of the HA genes of the swine viruses to those of human viruses circulating in consecutive years suggest a temporal appearance of the reassortant viruses. The selective pressures that drive the acquisition of different HA genes is unknown, but it is apparent that only progeny containing the triple-reassortant internal gene complex emerge from these reassortment events. It is also possible that reassortment with human strains is not occurring but, instead, that the HA heterogeneity has arisen through antigenic drift in pigs. Analysis of swine H3N2 viruses in The Netherlands and southern China showed that antigenic drift occurs. Phylogenetic analysis of these viruses, however, showed that swine and human viruses display diverging lines of evolution (12, 19). The triple-reassortant variants described in the present study do not form distinct lineages, and thus they appear to have arisen through reassortment rather than through antigenic drift.

It remains to be seen whether a particular triple-reassortant strain will predominate in U.S. swine or whether all the antigenic variants will cocirculate. The continual reassortment of swine viruses with this internal gene complex with circulating human strains has implications for both the future surveillance of U.S. swine herds and for vaccine efficacy. Additionally, the emergence of the triple-reassortant-like antigenic variants may mean that the prevalence of viruses of this genotype was underestimated in this study. If human-swine virus reassortment continues, it may be necessary to include current human H3N2 viruses and antisera in swine surveillance in addition to the index swine H3N2 viruses. It is uncertain how protective a vaccine produced from the 1998 triple-reassortant viruses will be against the newer isolates. Antiserum to Sw/TX/98 has only limited antigenic cross-reactivity to A/Sw/CO/23619/99 in HI tests, and the efficacy afforded by vaccination with the heterologous antigen may be limited. Certainly, if all of the triple-reassortant subgroups continue to cocirculate, a multivalent H3N2 vaccine may be needed.

Although the triple-reassortant viruses were not isolated until 1998, they may have been present in pig populations before this time. The increase in the frequency of H3N2-seropositive swine from 1.1 to 8.0% between 1989 and 1997 supports this hypothesis. On the basis of the sequences of the HA genes, the initial triple-reassortant viruses were most closely related to human viruses circulating in the 1995 era (39), and it can be postulated that the viruses entered the swine population around the same time. We propose two scenarios that are consistent with the slight increase in the seroprevalence of H3N2 viruses between 1989 and 1997. The first scenario is that a human H3N2 virus entered the pig population around 1995 and thus obtained gene segments of the swine and avian lineages. During the next few years, this virus circulated at undetectable levels in swine populations. Around 1998, this virus, either through mutation or simply by obtaining a critical density, caused disease in pigs and began to spread rapidly through swine herds in North America. The second possible scenario is that a human virus of the 1995 era, either alone or in conjunction with the swine or avian genes, circulated at low levels in pig populations. Upon acquiring the full complement of reassortant genes (i.e., swine, avian, or both), the virus became better adapted to swine and rapidly spread. Further analysis of archival sera and genetic characterization of recent classic

swine H1N1 viruses may help determine the evolutionary steps that led to the emergence of these viruses.

The establishment of the triple-reassortant virus in the United States has implications for both swine and human health. The transmission of H1N1 and H3N2 swine influenza viruses to humans has been documented (9, 11, 23, 26, 34, 37, 38). Although some of these interspecies transmissions have resulted in human fatalities, there has been limited human-to-human spread and no stable lineages have been established. As the distribution of the triple-reassortant virus increases, the contact between infected pigs and humans will increase correspondingly. The swine H3N2 viruses are antigenically similar to recent human viruses and therefore pose little direct threat to the human population. However, the potential exists for the transfer of the avian-like PA and PB2 genes to human viruses. All recent human pandemics have been caused by viruses containing an avian-like PB1 gene, suggesting that this gene may be important in the establishment of novel interspecies reassortants (reviewed in references 35 and 36). Although there are no such data implicating PA and PB2 genes, the previous documentation of the transfer of avian influenza virus genes to human viruses via the pig (9) suggests that swine industry workers should be monitored for indications of infection with triple-reassortant viruses.

Because of the presence of H3N2 viruses in U.S. swine, surveillance for the emergence of novel reassortant viruses should be performed. In the present study, 9.8% of tested animals had antibodies to both H1N1 and H3N2 viruses, a finding indicating the possibility of mixed infection. Reassortant viruses resulting from mixed H1N1 and H3N2 infection of swine have been isolated in areas where these two viruses cocirculate (3, 5, 13). Continued surveillance of North American swine herds should be done to ascertain which of the triple-reassortant viruses will remain circulating and to detect the emergence of new and potentially pathogenic virus strains.

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